Development of the Tubotympanum in the Rat

Jef J.S. Mulder, MD; Wim Kuipers, PhD; Theo A. Peters, BSc; Edith L.G.M. Tonnaer, PhD; Frans C.S. Ramaekers, PhD

Objective: To investigate the relationship between the anatomical maturation of the middle ear and that of the eustachian tube and paratubal muscles in the rat. Design: Wistar rats ranging from gestational day 12 to postnatal day 40 were used. Methods: Tissue specimens were examined with routine light microscopy and electron microscopy. Epithelial differentiation was studied immunohistochemically with antibodies to different cytokeratins. Results: The epithelial lining of the tubotympanum showed differentiation-related cytokeratin expression throughout the whole developmental period. The mucociliary epithelium reached mature features around birth. A dorsal extension and its framing cartilage started forming around 5 days after birth. This extension became lined by stratified nonciliated epithelium and attained maturity around 10 days after birth concurrently with the attachment of the dilatory muscles. This process was immediately followed by aeration of the middle ear cavity. Conclusions: The continuous expression of cytokeratins demonstrates that the epithelial lining of the tubotympanum is only derived from the embryonal endoderm. Furthermore, this study confirms that the eustachian tube shows a two-stage postnatal development. First, the mucociliary system matures, providing protection/clearance when the animal starts respiration and swallowing. Subsequently, the dorsal part attains maturity. The features of the epithelial lining of the dorsal part of the eustachian tube and the coincidence of the maturation of this part with the attachment of the dilating muscle fibers and the aeration of the middle ear indicates that this part provides ventilation. These findings support the authors’ hypothesis that different parts of the eustachian tube serve different purposes: clearance, protection and ventilation. Key Words: Eustachian tube, development, middle ear, epithelium, musculature, rat.


INTRODUCTION

The main functions of the eustachian tube are ventilation, protection, and clearance of the middle ear. To serve these functions, the eustachian tube is lined by mucociliary epithelium and is provided with paratubal muscles. Although it is generally accepted that the tensor veli palatini is the primary dilator of the eustachian tube, the precise roles of the epithelial lining and the paratubal muscles in these different functions are still a matter of debate.

Developmental studies have contributed significantly to our understanding of the relationship between anatomical maturation of the middle ear structures and auditory function. However, these studies have not addressed the relationship between the maturation of the middle ear structures and that of the eustachian tube mucosa and accessory structures, including the paratubal muscles.

In previous studies on the epithelium and musculature of the adult eustachian tube of the rat we established that the eustachian tube was not exclusively lined by mucociliary epithelium. It appeared that only the ventral part was lined by mucociliary epithelium, while the dorsal part showed the presence of squamous epithelium. On the basis of the spatial distribution of the different epithelia and the insertion of the dilating muscles in the dorsal part of the tube, a hypothesis was formulated concerning functional separation with respect to clearance/protection and ventilation. A similar hypothesis has recently been proposed for the eustachian tube in humans.

The aim of the present developmental study was to investigate the relationship between the anatomical maturation of middle ear and the maturation of the epithelial lining of the eustachian tube and paratubal structures to further test this hypothesis. In addition to routine histological techniques and electronmicroscopy, the differentiation of epithelial cells was studied immunohistochemically with antibodies against various cytokeratin polypeptides. Cytokeratins are intermediate filament proteins that are exclusively present in epithelial cells. The expression pattern of the 20 different subtypes depends on the type of epithelium, the stage of development, and the stage of differentiation.
MATERIALS AND METHODS

This study was performed on Wistar rats throughout a developmental period from gestational day 12 up to 40 days after birth. The day of the vaginal plug was considered day 1. Fetuses (aged 12, 14, 16, and 18 after conception) were removed by cesarean section and decapitated. Young rats of 1, 3, 5, 8, 10, 12, 15, 20, and 40 days were killed by decapitation. Four ears were used at each age for light microscopy and immunohistochemical studies. The whole head of the fetuses was processed, whereas a tissue specimen containing the middle ear, eustachian tube, and adjacent structures was dissected from the young rats. For electron microscopy, small tissue specimens were dissected from animals of 18 after conception and older, containing various parts of the epithelial lining of the developing tubotympanicum.

**Light Microscopy**

For light microscopy the specimens were fixed in phosphate-buffered (0.1 M; pH 7.4) glutaraldehyde (2.5%). After dehydration, the tissues were embedded in glycol methacrylate (GMA). All postnatal specimens were decalcified in EDTA (10%, pH 7.4) after fixation. Serial sections (2 μm) were made in a coronal or a sagittal plane. Every 10th section was stained with toluidine blue. From 18 after conception onward sections were also stained with alcian blue-PAS.

**Electronmicroscopy**

The tissue was fixed in phosphate-buffered (0.1 M; pH 7.4) glutaraldehyde (2.5%), decalcified in a solution containing phosphate buffer (0.1 M; pH 7.4) glutaraldehyde (1.25%), and EDTA (8%). After washing in phosphate buffer and postfixation in OsO4 (1%), the specimens were dehydrated and embedded in epon.

**Immunohistochemistry**

For immunohistochemistry the specimens were immediately frozen in liquid nitrogen. Specimens from animals of 8 days after birth and older were decalcified in a solution containing tris-hydrochloride buffer (0.1 M; pH 7.4), EDTA (10%), and polyvinyl pyrrolidone (7.5%) prior to freezing. Cryostat sections (7 μm) were made in coronal and sagittal planes. Sections were placed on poly-L-lysine-coated slides and processed for immunohistochemistry with a panel of antibodies to various cytokeratins and to vimentin, according to the indirect immunoperoxidase technique as reported previously. Ten different monoclonal antibodies (Mabs) against individual cytokeratins were applied in this study and one against vimentin. The Mabs are characteristic of the various types of differentiation: E2 (cytokeratin 8); CK16-2 (CK16); LIP2 (CK19); RCK 166 (CK7) of simple epithelial cells; IC7 (CK13) of stratification and 6B10 (CK3) of stratification and pseudostratification; RCK 107 (CK4) of basal cells and V9 of vimentin, the intermediate filament protein of mesenchymal tissues. For source and further specifications, see Vennix et al.

RESULTS

**Light Microscopy and Electronmicroscopy**

At gestational day 12 the first pharyngeal pouch consists of a small lateral extension of the pharynx, which is lined by endoderm. The lateral end of this extension is in close contact with the ectoderm of the first pharyngeal cleft, the primordium of the external ear canal (Fig. 1A). At 14 days after conception, the first pharyngeal pouch has transformed into a flattened tube, the tubotympanic recess. The recess is now separated from the primitive ear canal by a large mass of mesenchymal cells. By gestational day 16, the lateral part of the tubotympanic recess has expanded along the inner ear (Fig. 1B). The recess is lined dorsally by flat, one-layered epithelium and ventrally the epithelium consists of several cell layers (Fig. 1D). In the mesenchyme of the tympanal part, primordia of the ossicles can be distinguished (Fig. 1B).

At gestational day 18 the eustachian tube is clearly distinguishable from the primary middle ear cavity, by narrowing of the tubal part (Fig. 1C). The tubal part is lined by pseudostratified columnar epithelium (Fig. 1F) and electronmicroscopy shows the presence of developing ciliated and secretory cells. In the mesenchyme surrounding the pharyngeal part accumulations of myoblasts of the paratubal muscles can be distinguished (Fig. 1F). The primary middle ear cavity has further expanded up to the area of the round window niche and consists of a flattened pouch (Fig. 1C), which is lined by cuboidal and flat epithelium (Fig. 1E).

Developing ciliated and secretory cells can be distinguished in the presumptive ciliated tracts (Fig. 1E).

At 1 day after birth, the eustachian tube is funnel-shaped and still fully membranous. It is widest at the nasopharyngeal orifice (Fig. 2A) and gradually narrows laterally (Fig. 2B). The epithelial lining consists of mature ciliated epithelium and numerous secretory cells (Fig. 2A and B), showing secretory activity. On the caudal side of the eustachian tube, the large mixed gland has started to develop (Fig. 2A).

In the area of the nasopharyngeal segment the various paratubal muscles can clearly be distinguished (Fig. 2A). The levator veli palatini muscle (LVPM) is situated ventrally from the eustachian tube and its fibers insert into the caudal part of the soft palate. Muscle fibers of the medial bellies of the salpingopharyngeus muscle (SPM), s2 and s3, are attached to the dorsal and ventral wall of the eustachian tube, respectively, and course caudally to their insertion in the caudal lateral wall of the nasopharynx. At this age fibers of the lateral belly (s1) of the SPM (Fig. 2A) insert into the raphé at the skull base, but they do not have contact with the eustachian tube. The same applies to the fibers of the tensor veli palatini muscle (TVPM). They are connected with the palatal aponeurosis, but there is no contact with the eustachian tube (Fig. 2B). The primary middle ear cavity is still slit-like and is lined with flattened and low columnar epithelium. In the developing mucociliary tracts ciliated cells and secretory cells show the same mature features as in the eustachian tube and secretions are present in the lumen in this area.

By postnatal day 5, the eustachian tube is lined by mucociliary epithelium (Fig. 2C), but the epithelium in the dorsal part reveals stratification (Fig. 2E) along its whole length. In the mesenchyme, bordering the dorsal wall of the future intermediate membranocartilaginous and the lateral osseous tympanic segment, the tubal cartilage has started to develop concurrently with dorsal extension of the lumen (Fig. 2C). The number of muscle fibers and their thickness and length have increased, but the TVPM and the lateral belly (s1) of the SPM did not have any contact with the eustachian tube (Fig. 2C).

At this age, the mixed tubal gland shows an increased number of PAS-positive acini and secretory ducts, which are filled with secretions and open into the tubal lumen.

In the middle ear, the mesenchyme that fills the main part of the middle ear cavity has changed into myxo...
omatous vacuolated tissue with scattered fibroblasts and bundles of collagenous fibers. The ossicles and otic capsule reveal starting ossification.

At postnatal day 10 the intermediate segment of the eustachian tube, which shows a crescentic shape in cross-section (Fig. 2D) owing to the formation of the dorsal extension, is dorsally framed by a C-shaped cartilage. The lateral belly (s1) of the SPM has now become connected by a tendon to the tip of the caudal lamella of the cartilage (Fig. 2D, F). Also the TVPM shows its adult attachment. A minor number of the fibers insert into the fibrous tissue ventrally from the tip of the rostral lamella of the cartilage. The major part of the fibers is attached to the surrounding bone (Fig. 2D).

The dorsal lumen is lined by stratified epithelium (Fig. 2F) that shows locally varying features. The main part consists of stratified squamous epithelium (Fig. 3A), but areas with stratified cuboidal epithelium and stratified columnar epithelium with a superficial layer of columnar cells (Fig. 3B) are also observed. The luminal surface shows numerous microvilli and the apical part of the squamous cells bordering the lumen contains many secretory vesicles (Fig. 3C).

The mixed gland has obtained its adult size and numerous excretory ducts open into the lumen of the ventral part.

The myxomatous tissue, which obliterates the middle ear cavity (Fig. 4A), shows severe disintegration. In the external meatus, the skin is apposed to the epidermis of the tympanic membrane (Fig. 4A). At postnatal day 12 the middle ear has completely become aerated and the external meatus has opened.

At postnatal day 15 the external ear canal and the middle ear cavity have attained adult features and the osicular chain is fully ossified (Fig. 4B). In the eustachian tube the area occupied by stratified squamous epithelium and the number of cell layers have increased.

During further development, the shape of the eustachian tube and the features of the epithelial lining do not change, but the size, especially of the ventral membranous part, further increases up to the end of the observation period of 40 days.

**Immunohistochemistry**

At gestational day 12 the epithelium of the first pharyngeal pouch and the pharynx show homogenous stain-
Fig. 2. Sagittal sections showing cross-sections of the eustachian tube at different postnatal ages. (A) Pharyngeal segment lined with mucociliary epithelium at postnatal day 1. g = tubal gland; l = levator veli palatini muscle; s1 = lateral and s2 and s3 medial belies of salpingopharyngeus muscle. (Original magnification × 55.) (B) Future intermediate membranocartilaginous segment lined with mucociliary epithelium at postnatal day 1. l = levator veli palatini muscle; t = tensor veli palatini muscle. (Original magnification × 45.) (C) Future intermediate membranocartilaginous segment showing formation of dorsal extension (d) at postnatal day 5. c = cartilage; g = gland; l = levator veli palatini muscle; s1 = lateral belly of salpingopharyngeus muscle; t = tensor veli palatini muscle. (Original magnification × 45.) (D) Mature intermediate membranocartilaginous segment at postnatal day 10. c = cartilage; d = dorsal extension; g = gland; l = levator veli palatini muscle; s1 = lateral belly of salpingopharyngeus muscle; t = tensor veli palatini muscle. (Original magnification × 45.) (E) Detail of epithelium of dorsal extension (d) in C showing irregularly arranged stratified epithelium. (Original magnification × 300.) (F) Attachment of lateral belly of salpingopharyngeus muscle (s1) to ventral tip of dorsal lamina of c-shaped cartilage (c) at postnatal day 10. d = dorsal extension; g = gland. (Original magnification × 190.) All micrographs rostral: left margin; dorsal: top. Toluidine-blue stain.

ing with the simple epithelium-related markers Ck 18-2 (Ck18), E2 (Ck8), and LP2K (Ck19). This cytokeratin profile is also found at gestational day 14, but at this age the simple epithelium-related Ck7 (RCK 105) and the stratification marker Ck4 (6B10) are focally expressed at the future nasopharyngeal ostium.

At gestational day 16 the expression of Ck7 and Ck4 has further increased, whereas the basal cell marker RCK 107 (Ck14) shows heterogenous expression. By gestational day 18 Ck14 is expressed in the whole cellular layer. The stratification marker 6B10 (Ck4) is heterogeneously expressed in nearly the whole epithelial lining of both the eustachian tube and middle ear. This expression pattern does not fundamentally change in the mucociliary epithelium in the ventral part of the eustachian tube and in the ciliated tracts in the middle ear cavity up to maturity. In the adult this epithelium shows homogenous expression of Ck8, Ck18, and Ck19, whereas Ck7 and Ck4 are heterogeneously expressed. Ck14 is expressed in all basal cells. The cytokeratin profile of the flat squamous epithelium of the middle ear cavity does not significantly differ from that of the mucociliary epithelium.

In the dorsal part of the eustachian tube the cytokeratin profile changes from postnatal day 5 onward concurrently with the differentiation into stratified epithelium. A survey of the postnatal changes of the expression
of the simple epithelium-related Ck19 (LP2K) and the stratification marker Ck13 (IC7) in the intermediate segment is shown in Figure 5. By postnatal day 10 the stratification markers Ck13 (IC7) (Fig. 5E) and Ck4 (6B10) are expressed suprabasally in the stratified squamous epithelium of the dorsal part, whereas the simple epithelium-related markers Ck7, Ck8, Ck18, and Ck19 (Fig. 5D) are expressed to a varying extent in the stratified cuboidal and columnar epithelium.

At postnatal day 16 the area reacting with the stratification markers IC7 (Ck13) (Fig. 5E) and 6B10 (Ck4) has increased. The basal cell marker Ck14 (RCK 107) is expressed in the basal cells and in nearly all suprabasal cells of these stratified epithelia (Fig. 5F). This cytokeratin profile does not change up to 40 days after birth.

During early development, expression of vimentin is only found in the mesenchyme, but from gestational day 18 vimentin expression becomes apparent in scattered cells of the epithelial lining of the tubotympanic recess. During further maturation, the number of vimentin-positive epithelial cells, identified as ciliated cells, increases from postnatal day 6. All ciliated cells in the eustachian tube and middle ear cavity react with the vimentin antibody (Fig. 5I). In the stratified epithelium of the dorsal part of the eustachian tube, vimentin-positive dendritic cells can occasionally be observed.

Throughout the whole developmental period, the cytokeratin profile of the nasopharyngeal epithelium is similar to that of the mucociliary epithelium of the eustachian tube, but coexpression with vimentin is absent.

DISCUSSION

The present study demonstrates that the whole epithelial lining of the tubotympanum is derived from the endodermal lining of the first pharyngeal pouch and confirms the electron microscopic observations made by Hilding et al. Especially the expression of cytokeratins throughout the entire developmental period provides conclusive evidence that the mesenchyme does not contribute to the epithelial lining, as was stated by Schwartzbart and Marowitz and Porubsky.

During development, the epithelium shows a differentiation-related cytokeratin expression. This expression pattern is in line with reports on comparable epithelia in other parts of the respiratory tract, as well as with the general rules formulated for these epithelia. A remarkable observation is the coexpression of cytokeratins and vimentin in ciliated cells in the adult mucociliary epithelium. This phenomenon has often been reported during the early development of several epithelia.
However, in these epithelia, vimentin expression disappears during final maturation. Its appearance during the final maturation of the ciliated cells in the tubotympanum and its absence in the adjacent nasopharyngeal ciliated cells seem to refer to special properties of these cells in the tubotympanum. Such coexpression has also been reported in some columnar cells of the lung epithelium, but its significance is unknown.

The nonciliated epithelium in the dorsal portion of the eustachian tube has similar features to those of nonkeratinizing squamous epithelium in the esophagus regarding the expression of Ck4 and Ck13. However, in
the eustachian tube this epithelium shows expression of the basal cell Ck14 in nearly all cell layers. This demonstrates that the synthesis of Ck14 is not switched off when basal cells leave the basal cell layer and reflects a different differentiation pattern.

Throughout development, the shape of the tube and the features of the epithelial lining undergo comprehensive changes. Comparison of sections prepared with routine histology and cryostat sections obtained from freshly frozen specimens reveals a conspicuous difference with respect to the shape of the tube and the size of the lumen. This demonstrates that tissue processing by routine embedding causes severe shrinkage, which results in distortion of the anatomical proportions. Therefore, this method is not a reliable approach to study the patency of the eustachian tube.

The final maturation of the epithelium of the eustachian tube shows two consecutive steps. Around birth, both ciliated cells and secretory cells have reached maturity. A few days later acini of the developing tubal gland show secretory activity. A comparable observation has been made by Park et al. and Park and Lim in the murine eustachian tube. In addition, these authors showed that the secretions contained the antibacterial substances lysozyme and lactoferrin. These observations strongly suggest that the mucociliary system is functioning. This means that the protective function against ascending ascetions and microorganisms from the nasopharynx, begins when the animal starts respiration and swallowing. This protection is apparently independent of the functioning of the TVPM and lateral belly (s1) of the SPM, because these muscles do not have any contact with the tube at this time. However, a possible involvement of the IVPM and the medial bellies (s2 and s3) of the SPM, which are attached to the pharyngeal segment, cannot be excluded.

The second step, which starts around postnatal day 5, is the development of the dorsal extension and its cartilaginous framing. The lumen of the dorsal part becomes lined by squamous nonciliated epithelium. At postnatal day 10 these structures have obtained adult anatomical features. The SPM (s1) and TVPM, assumed to be the main dilatory muscles in the rat, are now attached to the ventral tip of the septum of the C-shaped cartilage and the dorsoventral membranous wall of the eustachian tube, respectively.

The character of the epithelial lining of the dorsal part of the eustachian tube and the coincidence of its maturation with the attachment of the dilatory muscles, immediately before the middle ear cavity becomes acrated, seem to confirm our assumption that this part of the eustachian tube serves middle ear ventilation. The present observations support our previous hypothesis that protection, clearance, and ventilation are spatially separated.

Based on the presence of nonsecretory epithelium in the dorsal part of the lumen and on the site of insertion of the TVPM, Sando et al. suggested the existence of a comparable spatial separation of these functions in humans. However, at present it is unknown whether the human eustachian tube undergoes similar stages of development in utero.

BIBLIOGRAPHY